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Original Article

The role and spectrum of *SLC26A4* mutations in Iranian patients with autosomal recessive hereditary deafnessNasrin Yazdanpanahi*, Mohammad Amin Tabatabaiefar[‡], Nader Bagheri[#], Fatemeh Azadegan Dehkordi[§], Effat Farrokhi[§] & Morteza Hashemzadeh Chaleshtori[§]

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Abstract

Objective: To determine the prevalence and types of *SLC26A4* mutations and the relevant phenotypes in a series of Iranian deaf patients. **Design:** A descriptive laboratory study. **Study sample:** One hundred and twenty-one families including 60 unrelated patients and 61 unrelated multiplex families with autosomal recessive deafness were included. In the 61 multiplex families, linkage was conducted for short tandem repeats (STRs) of the DFNB4. Selected individuals from the linked families and all of the 60 deaf individuals were subjected to sequencing of *SLC26A4*. **Results:** Seven out of the 61 (11.5%) families were linked to the locus which upon further inquiry led to identification of eight different mutations. Also, five out of the 60 (8.3%) patients were positive for the mutations. The *SLC26A4* mutations clarified in 9.1% (12 families) of total investigated alleles included: c.2106delG, c.65-66insT, c.881-882delAC, c.863-864insT, c.1226G>A, c.1238A>G, c.1334T>G, c.1790T>C, c.1489G>A, c.919-2A>G (IVS7-2A>G), c.1412delT, and c.1197delT. Six out of 12 (50%) families with mutations were confirmed to be Pendred syndrome (PS). **Conclusions:** The results probably suggest a high prevalence and specificity of *SLC26A4* mutations among Iranian deaf patients. Molecular study of *SLC26A4* may lead to elucidation of the population-specific mutation profile which is of importance in diagnostics of deafness.

Key Words: Deafness; genetic linkage; *SLC26A4*; mutation screening; enlarged vestibular aqueduct; goiter; Iran

Congenital hearing loss (HL) is globally the most prevalent sensorineural disorder which present in 1 of every 500 newborns (Morton & Nance, 2006). Genetic causes account for at least half of the etiology in cases with congenital HL. About 70% of genetic HL cases are non-syndromic (NSHL), where no other anomaly exists, while the remaining 30% include syndromic forms. So far, over 400 syndromic HL have been identified and Pendred syndrome (PS), an autosomal recessive sensorineural HL associated with enlarged vestibular aqueduct (EVA) and thyroid disease, is one of the most common examples (Pendred, 1896; Hilgert et al, 2009). NSHL can be inherited as autosomal recessive, dominant, mitochondrial, X- and Y-linked (Smith et al, 2005). Autosomal recessive HL is the most common type of HL and is prevalent in the consanguinity belt region of the world, including Iran, where consanguineous marriage is common (Hilgert et al, 2009; Smith et al, 2005). Thus

far, over 80 autosomal recessive (DFNB) loci have been mapped for ARNSHL (Autosomal recessive non-syndromic hearing loss) alone which proves extensive genetic heterogeneity of this trait (<http://www.hereditaryhearingloss.org>). *GJB2* mutations comprise 16–18% of ARNSHL in Iran (Chaleshtori et al, 2007; Najmabadi et al, 2005). Therefore, the rest of the etiology should be due to other loci (Tabatabaiefar et al, 2011).

SLC26A4 mutations cause either PS (OMIM#274600) or DFNB4 HL (OMIM#600791). It is responsible for about 4–10% of NSHL in different populations (Everett et al, 1997; Runge-Samuelson & Olivier, 2009). This gene is located at the DFNB4 locus on 7q31 chromosome (Everett et al, 1997). Enlarged vestibular aqueduct (EVA) and deafness are common features of both of the diseases. In addition to these signs, thyroid abnormality is seen in PS (Pendred, 1896). Among Caucasian patients with non-syndromic EVA, one

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Abbreviations

ARNSH	Autosomal recessive non-syndromic hearing loss
EVA	Enlarged vestibular aqueduct
HL	Hearing loss
NHS	Non-syndromic hearing loss
PS	Pendred syndrome
STR	Short tandem repeat

third of patients have been shown to carry two *SLC26A4* mutations, one third have one mutation and the rest have zero mutant allele (Pryor et al, 2005; Albert et al, 2006; Coyle et al, 1998).

The cost and inaccessibility of temporal bone study methods, phenocopy, and variability of goiter phenotype make clinical diagnosis of *SLC26A4* deafness difficult in many populations. Instead, molecular screening methods are a more accurate and proper strategy than clinical testing (Masmoudi et al, 2000 ; Napiointek et al, 2004; Fugazzola et al, 2007).

Different investigations have suggested that *SLC26A4* mutations are one of the most frequent causes of genetic HL in the world populations, including Iranians (Park et al, 2003; Kahrizi et al, 2009). Knowledge about ethnic and population differences in *SLC26A4* mutation prevalence can improve genetic strategies in diagnosis of the disease.

In many Iranian deaf patients, genetic cause of deafness remains undetectable. Up to now, most studies about the genetics of deafness in Iran have been performed on *GJB2*. Based on previous works (Kahrizi et al, 2009; Babanejad et al, 2012), we launched the current study in a cohort of Iranian HL families, with no *GJB2* mutations, to investigate *SLC26A4* mutations using molecular tools.

Materials and Methods

Sampling and DNA extraction

This study was approved by the institutional review board of Shahrekord University of Medical Sciences. Altogether 121 families including 60 unrelated patients (I1-I60), each of which from a multiplex family, and 61 unrelated multiplex families (IR1-61) were included in this study. All families had positive history of HL and were of Persian ethnicity. Also, for pathogenicity investigation 100 ethnically matched hearing controls were recruited.

A written informed consent had been provided for all the patients in our previous study (Chaleshtori et al, 2007). Pedigrees were drawn based on the filled-out questionnaire and interview with the family members.

All cases were negative for *GJB2* mutations in our previous study (Chaleshtori et al, 2007) and pedigrees suggested autosomal recessive mode of inheritance. There were at least two affected HL patients in pedigrees of all 61 families and 60 patients. At least one consanguinity loop was also detected in 109 of these pedigrees while for the rest of the families inbreeding was most likely the case.

For all cases, pure-tone audiometric test for air and bone conduction at frequencies 250 to 8000 Hz was performed to clarify HL severity as follows: mild (21–40 dB), moderate (41–70 dB), severe (71–95 dB) and profound (> 95 dB). For families with mutation, temporal bone CT-scan was conducted using Somatom Sensation Emotion 16-Slice Configuration (Siemens Medical Solutions, Erlangen, Germany) to clarify the vestibular aqueduct situation. EVA was considered when the diameter at the midway between the common

crus and the external aperture was equal to or more than 1.5 mm (Berrettini et al, 2005).

For assessing thyroid phenotype, T3, T4, and TSH hormones were measured using a chemiluminescent immunoassay (Berthold Technology-CSA, Germany) and ultrasonography was performed with a Sonoline G50™ ultrasound system (Siemens Medical Solutions, Erlangen, Germany). Results were interpreted based on sex and age.

Clinical phenotype of patients was investigated using temporal bone CT-scan, hormone assay and ultrasonography for most or all of HL family members. However, for known mutations, these tests were performed only for one patient of each family.

Genomic DNA for all available members of the families, patients and normal controls was extracted from 500 µl of peripheral blood using a standard phenole-chloroform method (Grimberg et al, 1989).

SLINK and STR selection

SLINK was calculated by the FastSlink (version 2.51) option of EasyLinkage (version 5.05) to evaluate power of the 61 pedigrees for linkage strategy (Lindner & Hoffmann, 2005). The families were screened by at least three informative STR markers before ruling out the linkage. In case evidence of linkage was observed, more additional markers were run to confirm the result. Altogether five STR markers for DFNB4 locus including D7S2459, D7S496, D7S2456, D7S2420, and D7S2425 were used in this study. They were selected based on NCBI MapViewer and UniSTS data.

STR genotyping and linkage analysis

Touch-down PCR was used for amplification of all STR markers. STR genotypes were assessed on 14% Polyacrylamide gel using transilluminator (Vilber Lourmat Deutschland GmbH, Germany). A subset of the results was confirmed by fragment analysis on an ABI 3730XL automated sequencer (Applied Biosystems).

Calculation of LOD score was combined with haplotype analysis for confirmation or exclusion of linkage. SuperLink (version 1.6) and SimWalk (version 2.91) options of EasyLinkage (version 5.05) were applied to obtain two and multipoint LOD score, respectively (Lindner & Hoffmann, 2005; Fishelson & Geiger, 2004). For LOD calculation, complete penetrance, autosomal recessive inheritance, disease-allele frequency of 0.001, no phenocopy, and equal recombination frequencies in both male and female were considered. Haplotypes were visualized by HaploPainter software (version 029.5) (Thiele & Nurnberg, 2005).

Mutation screening of SLC26A4

In seven linked families and all the 60 patients, sequencing of the *SLC26A4* gene was done to identify mutations. Twenty-one exons, exon-intron boundaries, and the promoter of the *SLC26A4* gene were amplified by standard PCR programs (assay conditions are available upon request). *SLC26A4* primers were designed using Oligo (version 6.7.1.0 National Biosciences Inc.) (Yazdanpanahi et al, 2012).

The PCR products were sequenced using an ABI 3730XL automated sequencer (Applied Biosystems). The resultant sequences were compared with *SLC26A4* reference sequence (NM_000441.1) using chromas (Version 2.33).

Pathogenicity investigation for variants c.2106delG, c.65-66insT, c.881-882delAC, c.863-864insT and c.1412delT was checked in our previous studies (Tabatabaiefar et al, 2010; Yazdanpanahi et al, 2012) and for the variant with unknown pathogenicity, c.1238A>G

(p.Gln413Arg) found in the current study. Study of pathogenicity was based on the nature of variants, evolutionary conservation of substituted amino acid, co-segregation in family members, and absence of variants in at least 100 hearing ethnically matched controls. PCR-RFLP-Polyacrylamide gel system were developed for each allelic variant (assay conditions are available upon request).

Results

Family data, SLINK and linkage analysis results

Altogether, 61 multiplex families (IR1-61) with 355 patients and also 60 patients, with positive history for deafness in their pedigrees (I1-I60), with congenital HL, and of Persian ethnicity were included in this study. SLINK average of the 61 families studied by linkage analysis was 3.33. There was at least one consanguinity loop within any of the pedigrees in 49 out of 61 (80%) families and all of the 60 HL patients. The average number of consanguinity loop within families was two.

Seven out of the 61 (11.5%) families were linked to DFNB4. SLINK, two-point and multi-point LOD score values for the seven linked families are shown in supplementary results, Supplementary Table 1 available online at <http://informahealthcare.com/doi/abs/10.3109/14992027.2014.944276>. The pedigree of one of the families (IR35) including the haplotypes is shown in Figure 1.

SLC26A4 mutation screening results

A total of 12 different *SLC26A4* mutations including c.2106delG, c.65-66insT, c.881-882delAC, c.863-864insT, c.1226G>A (p.Arg409His), c.1238A>G (p.Gln413Arg), c.1334T>G (p.Leu445Trp),

c.1790T>C (p.Leu597Ser), c.1489G>A (p.Gly497Cys), c.919-2A>G (IVS7-2A>G), c.1412delT, and c.1197delT were detected in 22 out of the 242 (9.1%) total studied alleles (Figure 2). In two families, only one heterozygous mutation was detected, two families were compound heterozygote and the remaining eight families were homozygote (See below). Five mutations (c.2106delG, c.65-66insT, c.881-882delAC, c.863-864insT, and c.1412delT), seen in four linked families, were reported as novel in our previous studies (Tabatabaiefar et al, 2010; Yazdanpanahi et al, 2012, 2013). The c.863-864insT and c.881-882delAC mutations were respectively identified in homozygous state in patients of the right and left sides of a large pedigree from Fars province, while was also found to be in compound heterozygous state in one patient from the pedigree (Yazdanpanahi et al, 2013). Three known mutations: c.1489G>A (p.Gly497Cys), c.919-2A>G, and c.1197delT were identified in three more linked families. Interestingly, c.1489G>A (p.Gly497Cys) and c.1197delT were found as compound heterozygous in a subset of patients from family IR35 (Figure 1). The four remaining mutations were found in five out of the 60 (8.3%) patients: c.1790T>C (p.Leu597Ser) was revealed in two different patients heterozygously. All of other mutations were homozygous (Table 1).

The results of our previously works suggested the pathogenicity role of the variants c.2106delG, c.65-66insT, c.881-882delAC, c.863-864insT, and c.1412delT (Tabatabaiefar et al, 2010; Yazdanpanahi et al, 2012, 2013). In the present study c.1238A>G (p.Gln413Arg) variant (with unknown pathogenicity) showed co-segregation with deafness in the related family members (supplementary results, Supplementary Figures 1, a, b, and 2 available online at <http://informahealthcare.com/doi/abs/10.3109/14992027.2014.944276>). The mentioned variant is a missense mutation resulting in

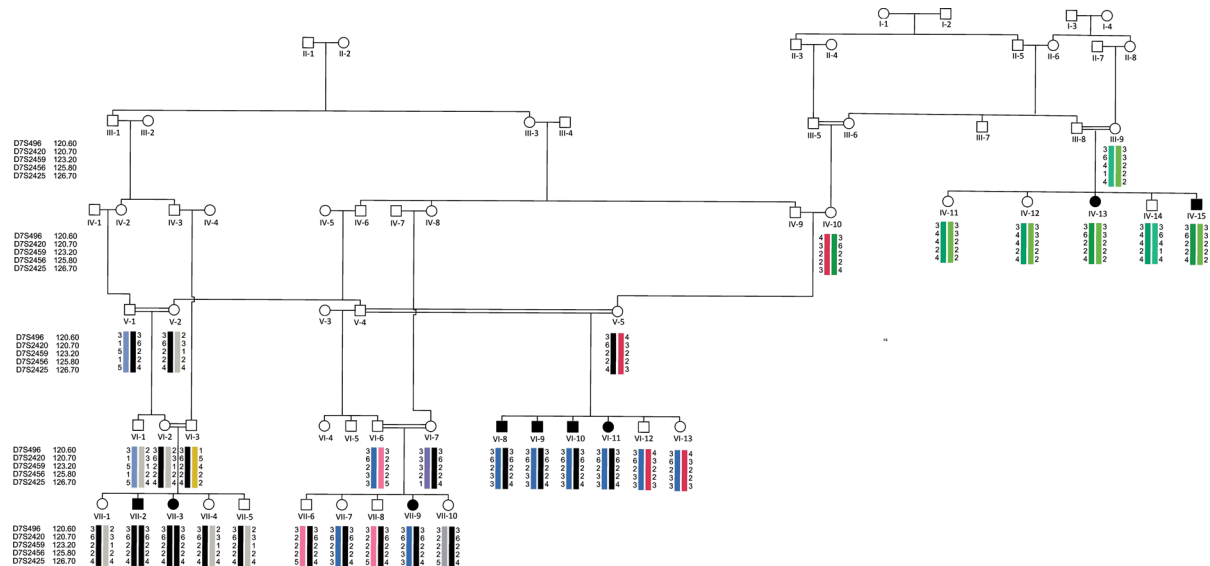


Figure 1. Haplotypes of the family IR35. Three different haplotype combinations were shown to be segregating with the disease. Maximum two-point and Multi-point LOD scores were 3.4 (for D7S2420) and 4.5 (for D7S2459), respectively. The order of the markers is based on LDB map. In one of the parents of individual 1 (V-4 from the left side pedigree) a cross-over event has occurred resulting in a novel haplotype distal to D7S2459 in him (not shown for him due to unavailability of the sample) as well as his progeny. Interestingly, all of the patients in the left side pedigree with either the original homozygous haplotype (3-6-2-2-4) or the combination of the original and the novel haplotypes (3-6-2-2-4/3-6-2-3-3) showed homozygous c.1197delT mutation. Two patients from the right side pedigree showed the combination of the original and a third haplotype (3-6-2-2-4/3-3-2-2-2). Both are compound heterozygous for c.1197delT/ c.1489G>A (p.Gly497Cys) which could serve as evidence that *SLC26A4* mutations are a frequent cause of hereditary deafness in the region (Khuzestan province, southwest Iran).

Table 1. Characterization of families with *SLC26A4* mutations.

Family	Number of affected patients in family	Age (years old)	Nucleotide change	Amino acid change	Exon	HL	Vestibular aqueduct	Thyroid	Province	Reference
IR1	2	(23,30)	c.2106delG/c.2106delG	p.Lys702AsnfsX19	19	Severe, Profound	EVA ¹	Normal, Goiter	Hormozgan	Yazdanpanahi et al, 2012
IR2	6	(21,24,27,32,35,40)	c.65-66insT/c.65-66insT	p.Ser23ValfsX64	2	Profound	EVA	Goiter	Chahar Mahal va Bakhtiari	Yazdanpanahi et al, 2012
IR3	6	(19,24,30,31,40,46)	c.881-882delAC/c.881-882delAC	p.His294GlnfsX35	7	Severe, Profound	EVA	Goiter	Fars	Yazdanpanahi et al, 2013
	2	(22,32)	c.863-864insT/c.863-864insT	p.Leu288PhefsX3	7	Severe, Profound	EVA	Goiter		
	1	(8)	c.881-882delAC/c.863-864insT	p.His294GlnfsX35/ p.Leu288PhefsX3	7	Moderate	EVA	Normal		
IR35	7	(23,25,27,28,30,35,36)	c.1197delT/c.1197delT	p.Cys400ValfsX32	10	Severe, Profound	EVA	Normal	Khuzestan	Everett et al, 1997;
	2	(23,34)	c.1197delT /c.1489G>A	p.Cys400ValfsX32/ p.Gly497Cys	10,13	Severe, Profound	EVA	Normal		Li et al, 1998
IR42	5	(34,42,43,45,47)	c.1412delT/1412delT	p.Leu471ArgfsX17	12	Severe, Profound	EVA	Goiter	Fars	Tabatabaie et al, 2010
IR51	4	(13,19,33,37)	c.1489G>A/1489G>A	p.Gly497Cys	13	Severe, Profound	NA ²	Normal	Guilan	Li et al, 1998
IR58	9	(18,21,25,26,28,31,36,42,45)	c.919-2A>G/919-2A>G	-	Intron 7	Severe, Profound	NA	Normal	Fars	Yong et al, 2001
I36	2	(33,35)	c.1226G>A/c.1226G>A	p.Arg409His	10	Profound	EVA	Goiter	Azerbaijan	Coyle et al, 1998
I50	2	(27,33)	c.1238A>G/c.1238A>G	p.Gln413Arg	10	Severe	EVA	Normal	Azerbaijan	de Moraes et al, 2013
I49	2	(25,28)	c.1334T>G/c.1334T>G	p.Leu445Trp	11	Profound	EVA	Goiter	Azerbaijan	Cremers et al, 1998
I44	3	(22,25,29)	c.1790T>C /-	p.Leu597Ser	16	Severe	EVA	Normal	Azerbaijan	Campbell et al, 2001
I47	4	(4,29,59,84)	c.1790T>C/-	p.Leu597Ser	16	Severe	EVA	Normal	Khorasan	Campbell et al, 2001

1. Enlarged vestibular aqueduct. 2. Not available.

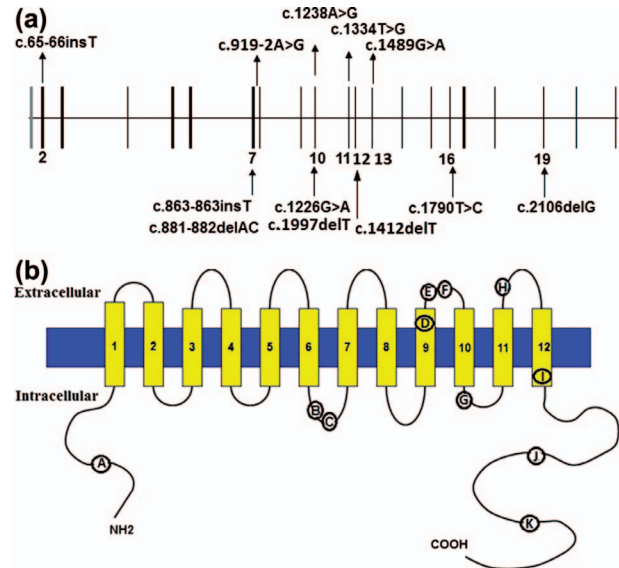


Figure 2. Location of *SLC26A4* mutations at the gene (a) and protein (b) levels. (b) A: c.65-66insT, B: c.863-864insT, C: c.881-882delAC, D: c.1197delT, E: c.1226G>A (p.Arg409His), F: c.1238A>G (Gln413Arg), G: c.1334T>G (p.Leu445Trp), H: c.1412delT, I: c.1489G>A (p.Gly497Cys), J: c.1790T>C (p.Leu597Ser), K: c.2106delG. (The mutation c.919-2A>G is intronic and does not change amino acid at the protein level).

the Glutamine to Arginine substitution (p.Gln413Arg). Glutamine is an uncharged polar amino acid but Arginine is a positively charged polar amino acid. Therefore, it is possible for the substitution to exert an effect on the structure, charge and function of the protein. Moreover, running Consurf server (<http://consurf.tau.ac.il>) revealed a high conservation score (8) for Glutamine at position 413 (Figure 3). This is evidence of high conservation degree of Glutamine 413 among orthologs.

Analysis of control individuals for c.1238A>G (p.Gln413Arg) revealed that one out of 143 individuals had this mutation heterozygously.

Clinical results

Clinical characterizations of families with *SLC26A4* mutations have been shown in Table 1. All the patients with mutation had severe to profound deafness, except one patient (age: 8 years) of IR3 family with moderate HL (Table 1). EVA was seen in all of the 10 families tested; no data were available for the two remaining families. Six out of the 12 (50%) families with mutations showed goiter. Phenotype of thyroid was sometimes variable even among the patients of the same family. Hypothyroidism was only detected in a 35-year-old patient of IR2 family.

Discussion

SLC26A4 has been reported as the second leading cause of autosomal recessive HL in many populations. The frequency and spectrum of *SLC26A4* mutations are different among various deaf populations (Table 2). So far, only little has been known about *SLC26A4* mutations in the Iranian population. Here, we could detect mutations in

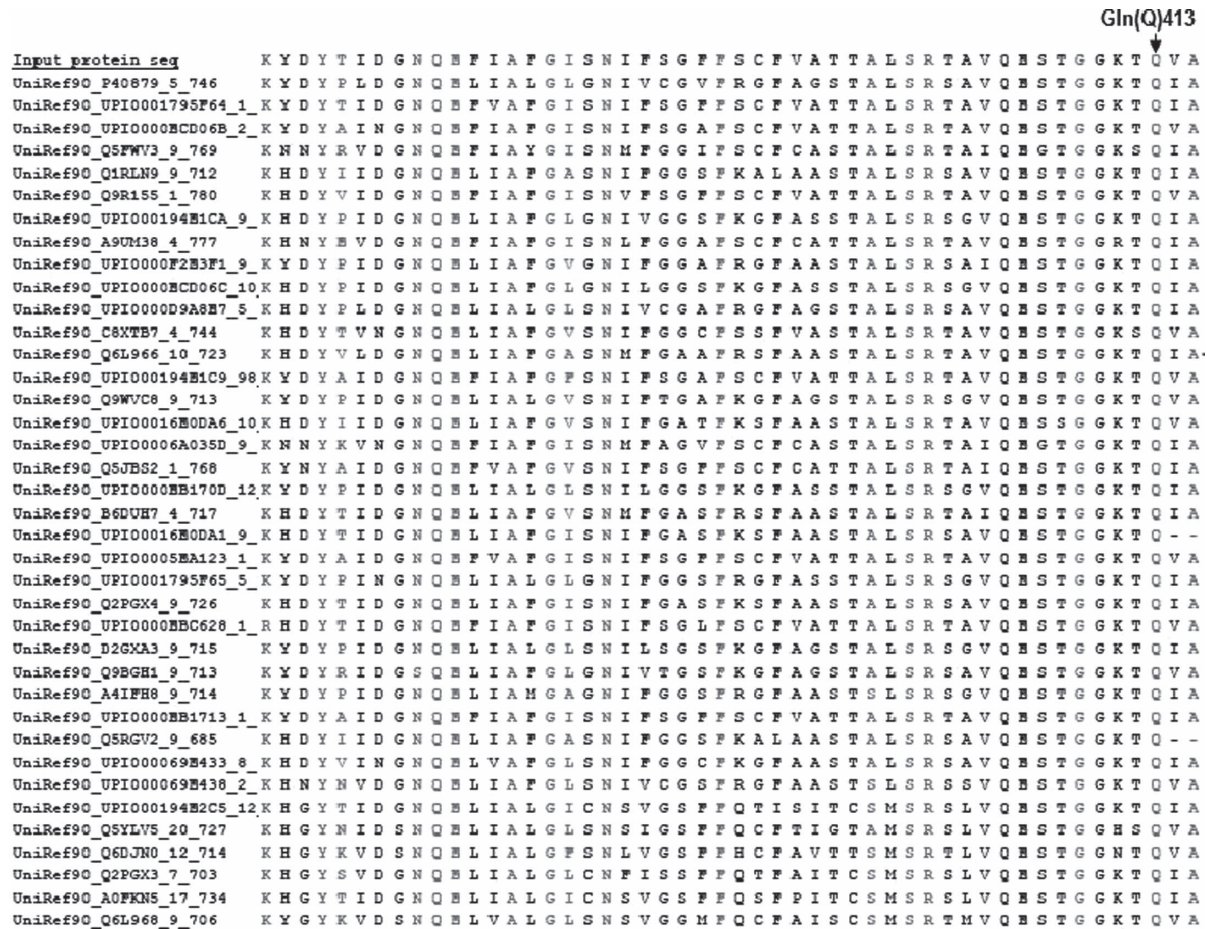


Figure 3. Result of Gln (Q)413 conservation investigation using consurf database. Gln (Q)413 amino acid is shown with arrow.

9.1% of total investigated alleles. The frequency of *SLC26A4* mutations among the Iranian HL patients has been reported to be 10% and 4.8% in two previous studies (Kahrizi et al, 2009; Babanejad et al, 2012). Thus, the results of our research with a relatively higher sample size, confirm the finding obtained by Kahrizi and colleagues (Kahrizi et al, 2009). The discrepancy between results obtained with our investigation and that performed by Babanejad and colleagues (Babanejad et al, 2012) might be due to ethnicity differences.

Notably, the result of the present study might be an over-estimation since they include heterozygotes with a second unidentified mutation. Detection of EVA in these heterozygotes patients

may be evidence of the presence of the second unidentified trans mutation. The missing cause is most likely a non-coding mutation or a large deletion in the coding region. Digenic mutations are also among the less likely alternative causes. Recently, digenic inheritance has been pinpointed in *KCNJ10/SLC26A4* (Yang et al, 2009) as well as *FOX11/SLC26A4* (Yang et al, 2007). Thus, these two genes could be interesting targets for further investigations.

c.2106delG, c.65-66insT, c.881-882delAC, c.863-864insT and c.1412delT are most probably pathogenic (Tabatabaiefar et al, 2010; Yazdanpanahi et al, 2012, 2013). The pathogenic role of mutation c.1238A>C causing the replacement of Glutamine in position 413

Table 2. *SLC26A4* mutations in different populations.

Population	No. of studied families (total alleles)	No. of mutated alleles (%)	The most frequent mutation (%)	Protein effect	Reference
US	255 (510)	35 (35/510 = 6.9)	c.1003T>C (5/35 = 14.3)	p.Phe335Leu	Dai et al, 2009
Spanish	67 (134)	36 (36/134 = 26.9)	c.1540C>A (6/36 = 17)	p.Gln514Lys	Pera et al, 2008b
Taiwanese	101 (202)	150 (150/202 = 74.2)	c.919-2A>G (115/150 = 76.7)	—	Wu et al, 2010
Chinese	302 (604)	131 (131/604 = 21.7)	c.919-2A>G (100/131 = 76.3)	—	Dai et al, 2009
Pakistanis	775 (1550)	112 (112/1550 = 7.2)	c.716T>A (34/112 = 30.3)	p.Val239Val	Anwar et al, 2009
Turkish	293 (586)	6 (6/586 = 1)	—	—	Tekin et al, 2003
Iranian	80 (160)	16 (16/160 = 10)	—	—	Kahrizi et al, 2009
	144 (288)	14 (14/288 = 4.8)	—	—	Babanejad et al, 2012
	121 (242)	22 (22/242 = 9.1)	—	—	This study

by Proline (p.Gln413Pro) has been formerly reported (Pera et al, 2008a). Pathogenicity study of c.1238A>G (p.Gln413Arg) in the present investigation suggests this variant is probably pathogenic. Five out of 12 (41.7%) mutations (c.2106delG, c.65-66insT, c.881-882delAC, c.863-864insT and c.1412delT) were reported in our previous studies for the first time (Tabatabaiefar et al, 2010; Yazdanpanahi et al, 2012, 2013). They may be unique to the Iranian population which should be further investigated. Exon 10 had the most number of mutations. We could detect two mutations (c.881-882delAC, c.863-864insT) in a single family, a proof for the high frequency of *SLC26A4* mutations among deaf families in Iran. Unlike *GJB2*, the *SLC26A4* gene has a bigger role in Asian than Caucasian populations (Park et al, 2003; Anwar et al, 2009; Dai et al, 2009) and p.L236P, p.T416P, p.E384G and IVS8 + 1G>A are the most frequent mutations of this gene in many populations (Coyle et al, 1998; Van Hauwe et al, 1998) but c.919-2A>G (IVS7-2A>G) and H732R mutations are more prevalent in East Asia (Park et al, 2003); c.919-2A>G (IVS7-2A>G) has also been found in one of the families of our study. Four mutations (p.Arg409His, p.Leu597Ser, p.Leu445Trp and c.1197delT) were identified both in this study and the previous investigation in the Iranian population (Kahrizi et al, 2009) and may be among the prevalent *SLC26A4* mutations in Iran. These results suggest a high allelic heterogeneity yet possible unique spectrum of *SLC26A4* mutations among Iranian patients. It might also be reasonable and cost-effective to initiate screening of *SLC26A4* with the search for these probably prevalent mutations.

In the current study six out of the 12 (50%) families with *SLC26A4* mutations had PS syndrome. In the previous studies in Iran, all of families with *SLC26A4* mutations were diagnosed with PS (Kahrizi et al, 2009; Babanejad et al, 2012). These results suggest that PS is probably a prevalent syndromic form of HL which has to be considered in molecular diagnostics in the Iranian deaf patients. Variable thyroid phenotype observed in this study among the patients with *SLC26A4* mutations has been reported by some investigators (Masmoudi et al, 2000; Napiontek et al, 2004; Fugazzola et al, 2007). This might be due to environmental and epigenetic factors as well as modifier genes. EVA was a constant feature among all the tested patients in this study.

Conclusion

From data presented here, it is apparent that *SLC26A4* mutations are likely to be the second most common cause of deafness after *GJB2* in Iran. Therefore it should be noted in molecular diagnosis of HL, especially when EVA and/or goiter are observed.

In summary, in the current study, we have addressed the frequency and spectrum of *SLC26A4* among Iranian subjects with hereditary HL. Our results confirm the role of the gene in pathogenesis of HL in Iran and might provide an insight into some prevalent mutations that are worth being prioritized in molecular diagnostics of HL in Iran. In view of the heterogeneity of the Iranian population, which consists of ethnicities of Persian, Azeri, Kurdish, Gilaki, Arab, Baloochi, Lur, etc., it would be interesting to study ethnic groups separately to better clarify the issue.

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Declaration of interest: The authors report no conflicts of interest.

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Supplementary material available online

Supplementary Table 1, Figures 1, a, b, and 2.